

## Postprint of: Plant Cell and Environment 39(11): 2498-2514 (2016)

**Title: Effects of different arbuscular mycorrhizal fungal backgrounds and soils on olive plants growth and water relation properties under well-watered and drought conditions**

**Running head:** Effects of AM fungal backgrounds on olive plants

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## Abstract

The adaptation capacity of olive trees to different environments is well recognized. However, the presence of microorganisms in the soil is also a key factor in the response of these trees to drought. The objective of the present study was to elucidate the effects of different arbuscular mycorrhizal (AM) fungi coming from diverse soils on olive plant growth and water relations. Olive plants were inoculated with native AM fungal populations from two contrasting environments, i.e. semi-arid – Freila (FL) and humid – Grazalema (GZ) regions, and subjected to drought stress. Results showed that plants grew better on GZ soil inoculated with GZ-fungi, indicating a preference of AM fungi for their corresponding soil. Also under these conditions, the highest AM fungal diversity was found. However the highest root hydraulic conductivity ( $L_{p_r}$ ) value was achieved by plants inoculated with GZ fungi and growing in FL soil under drought conditions. So, this AM inoculum also functioned in soils from different origins. Nine novel aquaporin genes were also cloned from olive roots. Diverse correlation and association values were found among different aquaporin expressions and abundances and  $L_{p_r}$ , indicating how the interaction of different aquaporins may render diverse  $L_{p_r}$  values.

Keyword index: Aquaporins, Arbuscular mycorrhizal fungi, Drought, *Olea europaea*, Root hydraulic conductivity

## Abbreviations list

AM: Arbuscular mycorrhiza  
FL: Freila (arid location)  
FW: Fresh weight  
 $g_s$ : Stomatal conductance  
GZ: Grazalema (humid location)  
 $H'$ : Shannon biodiversity index  
HPFM: High pressure flow meter

1	IBA: indolbutyric acid
2	Kr: Root hydraulic conductance
3	L <sub>pr</sub> : Root hydraulic conductivity
4	OTU: Operational taxonomic unit
5	PIP: Plasma membrane intrinsic protein
6	RWC: Relative water content
7	S: Specific richness
8	TIP: Tonoplast intrinsic protein
9	ΦPSII: photochemical efficiency of photosystem II
10	
11	

## 1    **Introduction**

2    Olive trees (*Olea europaea* L.) are among the most important crops for the Mediterranean  
3    area. They grow in very diverse environments (Parodi 1978), and are known for their  
4    resistance to water stress, especially during summer, when they face scarce precipitation  
5    and high temperatures (Connor 2005). Nevertheless, their response to water stress has been  
6    shown to be quite unpredictable, as their intraspecific genetic diversity is high (Guerfel et  
7    al. 2009). Even if olive tree is considered as a drought resistant species, the number of  
8    irrigated cultivars is increasing (Carr 2013), and the balance between irrigation and  
9    productivity is a major issue as water is scarce in most countries growing this crop. With  
10   the current prediction of a decline in water availability and an estimation of    crop  
11   production decline for the next years, the selection of olive trees with enhanced drought  
12   tolerance is needed to maintain and even improve their productivity.

13        The effects of drought on plants vary amongst species considered, although one of  
14   the first responses of plants to the lack of water within the soil profile is to adjust their  
15   internal water balance by closing stomata (Gomez-del-Campo, 2007; Schachtman and  
16   Goodger, 2008), and by regulating their root hydraulic conductivity (Maurel et al., 2008;  
17   Aroca et al., 2012). Even though the regulation of root hydraulic conductivity has been  
18   mainly attributed to the action of aquaporins (Luu and Maurel 2005, Maurel et al. 2015),  
19   root and leaf osmotic adjustment as well as root architecture may be also playing a very  
20   important role in water uptake under prolonged period of drought stress. Three aquaporins  
21   have been described in olive trees, i.e *OePIP1;1*, *OePIP2;1*, and *OeTIP1;1* (Secchi et al.  
22   2007, Lovisolo et al. 2007). As in another tree species, these aquaporins have been  
23   reported to be differently regulated according to the stress, and the intensity of the stresses  
24   (Secchi et al. 2007). Osmotic regulation in severely water stressed olive trees has been also  
25   described in experiments with these trees and may be a complementary strategy to

1 aquaporins in order to resist long-term exposure to soil water deficit (Chartzoulakis et al.,  
2 2000; Ennajeh et al., 2008). At the same time, Tataranni et al. (2016) found a close  
3 correlation between morpho-anatomical traits and  $L_{p_r}$  values in olive trees subjected to  
4 drought.

5         The use of AM fungi as relievers of the drought stress effects on plants has been  
6 studied for many years. AM fungi are believed to have a great capacity to resist fast  
7 environmental changes under drought conditions and long-term stresses, allowing the  
8 plants to have a wider range of possibilities to adapt and survive (Al-Karaki 1998; Smith  
9 and Read 2008; Ruíz-Lozano et al. 2012). Olive trees appear to be highly dependent on  
10 AM fungi under arid conditions (Mekalia et al. 2013), although the effect of the AM  
11 symbiosis on plants varies according to the AM fungal strain used and the plant cultivars  
12 (Binet et al., 2007), as well as to the soil chemical composition (Burns et al. 2015). AM  
13 fungi establishes an extensive hyphal network in the soil, mobilizing soil nutrients, mainly  
14 phosphorus and nitrogen (Bonfante and Genre 2010, Bompadre 2013) that would play a  
15 key role in plant survival under stress conditions. In general, AM fungi have been shown to  
16 increase plant resistance to drought (Azcón et al., 1996; Porcel et al. 2004), and to alleviate  
17 water stress (Sheng et al., 2008; Bárzana et al., 2012), while improving plant productivity  
18 (Navarro-Fernández et al., 2011; Abbaspour et al., 2012), and nutrient status (Farzaneh et  
19 al., 2011; Lee et al., 2012). Furthermore, AM symbiosis causes significant changes in  
20 aquaporin abundance and activity in host plants (Aroca et al., 2007, Jahromi et al. 2008,  
21 Bárzana et al., 2014). For example, a consistent diminution of PIP2 aquaporin  
22 phosphorylation has been observed in bean roots colonized by the AM fungus *Rhizophagus*  
23 *irregularis* under optimal conditions (Aroca et al. 2007; Benabdellah et al. 2009; Sánchez-  
24 Romera et al. 2016), suggesting less activity of such aquaporins (Maurel et al. 1995).  
25 These results support the idea that each aquaporin has a specific function under different

environmental conditions (Aroca et al., 2007; Jang et al., 2007; Calvo-Polanco et al. 2014a) and that each plant will respond differently to each AM colonizing fungus. Therefore, the regulation of root hydraulic conductivity ( $L_{pr}$ ) and aquaporin expression and abundance by AM symbiosis is far from being understood. In studies using the same plant and AM fungal species, the response of aquaporin expression to AM inoculation was different (Aroca et al. 2007; Sánchez-Romera et al. 2016), probably because the soil used was not the same. Also, El-Mesbahi et al. (2012) found that the response of  $L_{pr}$  and aquaporin expression to AM symbiosis depended on the soil  $K^+$  content. Also, different AM fungal species had different effects on aquaporin expression in soybean and lettuce plants (Porcel et al. 2006).

In the present study, we analyzed the response to drought stress of seven month-old olive plants (*Olea europaea* cv. Picual) grown in natural soils from two locations with contrasting climatology: Freila (FL), a Mediterranean location in the south of Spain with low average annual precipitation (380 mm), and Grazalema (GZ), whose average annual precipitation is high (2223 mm). Plants were inoculated with the native AM fungal population of each soil, including crossing of soils with non-autochthonous AM fungi. The plants were subjected to drought stress for four weeks. The objectives of the study were: 1) To study how different combinations of soils, AM fungal communities and water regime modify root hydraulic properties of olive trees including aquaporin expression and abundance, 2) to identify novel aquaporin genes in olive plants taking advantage of the OLEAGEN database (Muñoz-Mérida et al. 2013), and 3) to find out which physiological or molecular traits are potential more determinants of  $L_{pr}$  regulation.

## **Material and Methods**

### *Olive plants production and growth*

We used olive (*Olea europaea* L. cv. Picual) plants to test the effect of two contrasting natural soils from the Mediterranean area in Spain combined with the natural AM population from those two soils on plant tolerance to severe drought stress. This particular olive cultivar is known for its moderate drought tolerance, since it has been selected for rain fed conditions (Tugendhaft et al. 2016). Olive explants were produced from mature olive trees growing at FL (37°31'43"N, 2°54'34"W), a Mediterranean location in the south of Spain. Olive branches were removed and transported in a humid piece of cloth to a green house. Cuttings were produced as explained by Suarez et al. (1999). Briefly, 18 cm length and 4-6 cm diameter cuttings were treated with indolbutyric acid (IBA) 3500 ppm by immersing the cutting base for 10 seconds in a 1:1 (v:v) IBA hydro-alcoholic solution. The explants were immediately transferred to a mist propagation system for 90 days on a perlite substrate at 25 °C basal heating for rooting.

Once the explants were rooted, they were transferred into 2 L pots using two different natural soils; one originating from FL area, and another one from the GZ area (36°46'4"N, 5°21'57" W). These areas were chosen for their high olive oil production and their contrasting climatology. FL is a typical semi-arid Mediterranean location, with dry and hot summers, mild winters and low annual total precipitation (380 mm), while the GZ area has more a continental climate with cold winters and hot summers, and much higher total annual precipitation (2223 mm). Soil was collected from four different points (20 Kg at each point), spaced 10 m each other in each olive field. The soil from the four points was mixed later. Only Horizon-A was collected. The soils were sieved (50 mm) and sterilized by steaming for 1 h, for three consecutive days at 100°C. Plants were then transferred to a greenhouse at 22/18°C, 65% relative humidity, and were grown for seven months. Plants were irrigated with 50% modified Hoagland's solution once per week (Epstein1972): 2.5

1 mM KNO<sub>3</sub>, 0.5 mM KH<sub>2</sub>PO<sub>4</sub>, 2.5 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 23 µM H<sub>3</sub>BO<sub>3</sub>, 5 µM MnCl<sub>2</sub>, 0.3 µM ZnSO<sub>4</sub>, 0.2 µM CuSO<sub>4</sub>, 0.01 µM (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, 90 µM EDTA-Fe.

Inoculation was carried out at the time of planting. The plants growing in each soil were divided into three different groups and the inoculation treatments were applied as follows: 1) control-non-AM inocula, 2) natural AM fungal population from FL soil, and 3) natural AM fungal population from GZ soil. The AM fungal inocula from the natural soils were obtained by using the wet sieving and decanting technique (Navarro-Fernández et al. 2011). Briefly, 310 g of the corresponding 2 mm sieved soil (which is equivalent to 500 ml of each soil with the original texture) was suspended in 2 L of water. Then, the suspension was strongly stirred for sample homogenization and left for soil decantation. The supernatant was poured out through coupled sieves of 700 µm, 500 µm, 250 µm and 50 µm. This procedure was carried out twice with the same soil sample. Finally, the material from all sieves except from that of 700 µm was added to each pot in the planting hole, according to the corresponding treatment. Thus, the experiment consisted of twelve treatments arising from the combination of two different soils (FL and GZ), three AM inoculum (non-inoculated, native AM fungi from FL soil, and native AM fungi from GZ soils) and two water regime treatments (well-watered and drought stress conditions). Each treatment was composed of six plants.

#### *Soil analyses and watering regime*

Elemental analyses of the natural soils from FL and GZ and from olive leaves were carried out by an ICP plasma analyzer at the Instrumental Service of the Estación Experimental del Zaidín (CSIC). The results of soil analyses are presented in Table 1.

Six months after planting, plants from each fungal treatment were separated into two groups; half of them were well-watered (95% field capacity) and the other half



submitted to drought stress (55% field capacity). Soil moisture was controlled using a ML2 ThetaProbe (AT Delta-T Devices Ltd., Cambridge, UK), and after that, the water content of the soil was maintained by weighing the pots every day and replacing the water lost to recover the desired level of soil water content (Porcel et al. 2004). The drought treatment lasted four weeks.

#### *AM colonization rates and fungal mycelial length*

Mycorrhizal root colonization was estimated by analyzing four roots ( $n=4$ ) per treatment combination. Approximately 0.5 g of root tissues were cleared in 10% KOH and stained with 0.05% trypan blue in lactic acid (v/v). The extent of mycorrhizal colonization was calculated according to the gridline intersect method (Giovannetti and Mosse 1980).

Fungal mycelial length was determined as explained in Abbott et al. (1984). For the calculation of the total length of the mycelia, the following equation was used as:  $R = (\pi AN/2H) \times F_d \times F_{ps}$ , where  $R$  is the hyphal length,  $A$  is the area of the filter ( $m^2$ ),  $N$  is the number of intersections,  $H$  the total length of the reticulum used,  $F_d$  is the dilution factor, and  $F_{ps}$  the dry weight factor.

#### *Molecular identification of AM fungi colonizing the roots*

For AM fungal identification, genomic DNA was extracted from 120-140 mg of fine roots from each treatment combination by using the DNeasy Plant Mini Extraction Kit (Qiagen Inc., Mississauga, ON, Canada) following manufacturer's instructions. A nested polymerase chain reaction (PCR) approach was used to amplify a partial LSU rRNA gene region (approx. 370bp) of the AM fungal DNA from the root samples. The primer combinations LR1/FLR2 and FLR3/FLR4 were employed as explained in Gollotte et al (2004). All amplifications were performed on a Mastercycler Nexus PCR cycler

(Eppendorf, Hamburg, Germany) by using Canvax Biotech Taq polymerase (Canvax Biotech, Cordoba, Spain). PCR products were separated by gel electrophoresis on a 1% agarose gel in TAE buffer, and DNA was visualized under UV light after being stained with ethidium bromide.

Four to five replicates of both PCR amplifications were performed for each sample and the resulting amplicons pooled to yield a composite sample (Renker et al 2006). The mixed products from the second PCR were cloned into the p-GEM<sup>®</sup> T-Easy Vector (Promega, Madison, USA) and used to transform commercial DH5 $\alpha$  competent cells (Invitrogen, Carlsbad, USA), following the instructions of the manufacturer. Up to 40 clones were screened from each cloning reaction to check the correct length of plasmid inserts by colony-PCR using vector primers T7 (5'-TAATACGACTCACTATAGGG-3') and M13r (5'-GGATAACAATTCACACAGG-3'). At least 30 clones, containing inserts with the correct size, from each LSU rDNA library were sent for sequencing to GATC (GATC Biotech, Konstanz, Germany), using the vector primer M13-FP (5'-TGTAACGACGGCCAGT-3').

All sequences obtained were aligned and adjusted manually using the program BioEditversion 7.1.3 (<http://www.mbio.ncsu.edu/bioedit>). Sequence types or operational taxonomic units (OTUs) were defined in a conservative manner as clusters of closely related sequences with a level of pairwise similarity higher than 98%, except if different OTUs contained sequences from the same deposited isolate. Through BLAST (Basic Local Alignment Search Tool) on-line tool, a preliminary taxonomic classification was obtained for all defined OTUs. Clones of non-AM fungal origin were excluded from further analyses.

The abundance of each OTU in the different treatment combinations was represented as relative abundance in relation to the total number of AM fungal clones considered in each

clone library. It is assumed that each AM fungal type is amplified and cloned proportionally, being the abundance of each OTU as an approximate estimation of their proportion in the root. The Shannon biodiversity index ( $H'$ ) was used to evaluate the genetic diversity (hereafter AM fungal diversity) and calculated by the formula  $H' = -\sum (n_i/N) \ln(n_i/N)$ , where  $n_i$  represents the number of sequences belonging to each phylotype and  $N$  the total number of phylotypes (Shannon & Weaver, 1963). The phylotypes' Specific Richness ( $S$ ) was also calculated for each plant.

#### *Physiological parameters*

Root and leaf fresh weight were determined at the time of harvest in six ( $n=6$ ) plants per treatment combination. Relative height increments and relative number of leaves were determined from the measurements taken at the beginning and at the end of drought treatment.

Stomatal conductance ( $g_s$ ) was measured before harvesting, in the last-fully developed mature leaves with a portable AP4 Porometer (Delta-T Devices Ltd, Cambridge) three hours after sunrise. The photochemical efficiency of photosystem II ( $\Phi_{PSII}$ ) and leaf relative water content (RWC) were determined in six mature leaves per treatment combination ( $n=6$ ), using the same leaves as for  $g_s$ . For  $\Phi_{PSII}$ , we used a FluorPen FP100 (Photon Systems Instruments, Brno, Czech Republic), which allows the measurement of chlorophyll a fluorescence and hence plant photosynthetic performance. To determined RWC, mature, fully-developed leaves were excised from the main shoot, weighed ( $W_0$ ) and introduced into 15 ml centrifuge tubes (BD Falcon, Fisher Scientific) with a piece of wet cotton for 24 h at 4 °C. Leaves were weighed again ( $W_h$ ), dried at 75°C

for two days and then weighed ( $W_d$ ) a third time, and the relative water content was calculated as:  $RWC = (W_0 - W_d) / (W_h - W_d) \times 100$ . Leaf chlorophyll contents were extracted in 100% methanol and concentration calculated using the coefficients and equations reported in Lichtenthaler (1997).

Root hydraulic conductance ( $K_r$ ) was determined for six complete roots ( $n=6$ ), per each of the twelve treatments. We used a high pressure flow meter (HPFM, Dynamax, Inc., Houston) and the measurements were taken in the same plants used for  $g_s$ , between three to four hours after sunrise. Detached roots were connected to the HPFM and water was pressurized into the roots from 0 to 0.5 MPa in the transient mode to calculate root hydraulic conductance ( $K_r$ ; Calvo-Polanco et al. 2012). Root hydraulic conductivity ( $L_{pr}$ ) was determined by dividing  $K_r$  by the root volume (Calvo-Polanco et al., 2012).

### *Olive aquaporins identification*

For olive aquaporins identification, total RNA was isolated from roots by a phenol/chloroform extraction method followed by LiCl precipitation (Kay et al. 1987). cDNA was synthesized from 2  $\mu$ g of total RNA using oligo(dT)<sub>12-18</sub> as a primer and M-MLV as reverse transcriptase (Invitrogen). For the identification of aquaporins, cDNA was amplified by PCR using the degenerate primers from Park et al. (2010). PCR reactions were performed as described for AM fungal identification, except that the annealing temperature was changed to 60°C. After gel electrophoresis of the PCR products, visible bands of the expected size (approx. 400kb) were recovered from the gel, eluted with a QIAquick Gel Extraction Kit (Qiagen) and cloned as explained previously. To determine the sequences of the full-length cDNAs, we used the SRS database from the OLEAGEN consortium (Muñoz-Mérida et al. 2013).

Amino acid sequences predicted for the olive aquaporin genes were used for phylogenetic analysis along with those predicted for other plant aquaporin genes using MEGA4 software (Tamura et al. 2007). The reliability of the branches in each resulting tree was supported with 1,000 bootstrap resampling. Sequences generated in this study have been deposited at the European Molecular Biology Laboratory (EMBL) database under the accession numbers KT380900 to KT380908.

#### *Root aquaporin expression analysis*

Aquaporins expression was determined in the roots of three plants of each treatment combination (n=3). Total RNA was isolated as described above. DNase treatment of total RNA and reverse transcription were done following the instructions provided by the manufacturer (Quantitect Reverse Transcription KIT Cat#205311, Qiagen, CA). We used, for the root aquaporin expression analyses, the three known *O.europaea* aquaporins (*OePIP1;1* GeneBank accession no DQ202708, *OePIP2;1* GeneBank accession no DQ202709, and *OeTIP1;1* GeneBank accession no DQ202710), plus the new ones described in this study (*OePIP1;2* GeneBank accession no KT380904; *OePIP1;3* GeneBank accession no KT380905; *OePIP2;2* GeneBank accession no KT380900; *OePIP2;3* GeneBank accession no KT380903; *OePIP2;4* GeneBank accession no KT380908; *OePIP2;5* GeneBank accession no KT380901; *OePIP2;6* GeneBank accession no KT380906; *OeTIP1;2* GeneBank accession no KT3809902; and *OeTIP1;3* GeneBank accession no KT380907). The expression of the different aquaporins was determined using a real time quantitative PCR (iCycler-Bio-Rad, Hercules, CA) as explained in Calvo-Polanco et al. (2014a, b). We could not detect any expression from *OePIP1;1* and *OePIP2;1* in the roots of our olive plants. For the aquaporins analyzed, the annealing temperature was 58°C and the primers used are described in the Supplementary FileS1.

The specificity of the PCR amplification procedure was confirmed using a heat dissociation protocol (from 60 to 100°C) after the final cycle of the PCR. The relative abundance of transcription was calculated using the  $2^{-\Delta\Delta C_t}$  method (Livak and Schmittgen, 2001). To normalize aquaporin expression, we tested different olive housekeeping genes: actin, ubiquitin, and elongation factor. The elongation factor was the one chosen after RT-qPCR as it displayed stable mRNA levels throughout all treatments. Elongation factor-specific primers were used for standardization by measuring the expression of elongation factor gene in each sample. Negative controls without cDNA were used in all the PCR reactions.

#### *Proteins isolation and ELISA analysis*

Microsomes were isolated as described in Hachez et al (2006). For ELISA analysis, two micrograms of the protein extracts were processed as described in Calvo-Polanco et al. (2014a, b). We used, as primary antibodies (at a dilution of 1:1000), the two antibodies that recognize several PIP1 and PIP2 and three antibodies that recognize the phosphorylation of PIP2 proteins at their C-terminal region at Serine 280 (PIP2<sub>280</sub>), Serine 283 (PIP2<sub>283</sub>) and both at Serine 280 and 283 (PIP2<sub>280/283</sub>) as described in Calvo-Polanco et al. (2014a, b). A goat anti-rat IgG coupled to horseradish peroxidase (Sigma-Aldrich Co., USA) was used as secondary antibody at 1:10,000 for PIP1. Goat anti-rabbit IgG coupled to horseradish peroxidase (Sigma-Aldrich Co., USA) was used as secondary antibody at 1:10,000 for PIP2 and PIP2<sub>280</sub>, PIP2<sub>283</sub> and PIP2<sub>280/283</sub>. Protein quantification was carried out in three different independent root samples per treatment (n=3), replicated three times each. PIP2 antibodies antigens were aligned to see which *Olea europaea* aquaporins could be recognized by each antibody (Supplementary FileS2). The specificity of the PIP2 and

phosphorylated antibodies PIP<sub>2</sub><sub>280</sub>, PIP<sub>2</sub><sub>283</sub> and PIP<sub>2</sub><sub>280/283</sub> is described in Calvo-Polanco et al. (2014b).

#### *Statistics*

Data were analyzed using ANOVA with the Proc MIXED procedure in SAS (version 9.2, SAS institute Inc., NC, USA) together with the post-hoc Tukey's test to detect significant differences among all treatment means. When the ANOVA p-values for the three way interaction was not significant, we proceed to run t-test for the significant interactions. The aquaporin expression and abundance data, mycorrhizal colonization rates and length, stomatal conductance, together with the L<sub>p</sub><sub>r</sub> data were firstly sorted out using a principal components analyses (PCA) and posterior Pearson correlations to determine which of the aquaporins may have contributed to L<sub>p</sub><sub>r</sub> and if g<sub>s</sub> was correlated with L<sub>p</sub><sub>r</sub>. Pearson correlations were also calculated among L<sub>p</sub><sub>r</sub> and stomatal conductance and leaf N, P and K contents.

## **Results**

### *Soil conditions and plant growth*

GZ and FL soil analyses showed high pH values, between 8 and 9, and low electrical conductivity (Table 1). GZ soil was richer in nutrients (higher N, P, K and Ca) and also in organic matter which should favour plant growth. Significant lower values of N, P, K and S contents were observed in leaves of plants growing in FL soil, mostly in non inoculated plants (Table 2). Inoculation with AM fungi from FL soil increased P and K contents of

leaves of plants growing in FL soil (Table 2). The difference in K content between leaves from plants growing in both kinds of soils was only significant under well watered conditions (Table 2).

When plant growth was analysed using either leaf or root fresh weight (FW) as a parameter, the impact of the soil origin become clear as GZ soils supported larger growth ( $p<.0001$  and  $p=0.044$ , respectively) (Table 3). However, drought stress induced a significant reduction of the leaf FW ( $p=0.0008$ ), in both non-inoculated plants and plants inoculated with the FL-inocula growing in GZ soil (Table 3). On the other hand, root FW was significantly reduced by drought in all plants growing in FL soil, while no effect could be detected on GZ soil (Table 3). There was also a significant positive effect of the mycorrhizal inoculation in leaf FW ( $p=0.01$ ; Table 3).

Drought treatment also affected relative plant height ( $p=0.04$ ) (Figure 1B), as well as the relative number of leaves produced during the drought treatment ( $p<.0001$ ) (Figure 1A). Plants growing in GZ soil with the GZ inocula were the only ones that displayed a significant increase in their relative heights under well-watered conditions, while the relative height did not change in any of the other treatments considered (Figure 1B). For the relative number of leaves, drought caused a significant loss of leaves in the GZ soil plants in either non-inoculated plants and those inoculated with FL-AM fungi (Figure 1A). It is noteworthy that under these conditions, inoculation with GL-AM fungi prevented such decrease.

#### *Fungal mycelium length, root mycorrhization rates and fungal diversity*

The highest extension of external mycelia under well-watered conditions was found in plants growing in FL soil and inoculated with AM fungi from GZ (Figure 2A,  $p<.0001$ ). When drought was applied, the highest external fungal development was found in FL soils with FL AM fungi. In GZ soils, hyphae within the soil were longer in the presence of GZ



fungi, despite the water regime (Figure 2A). However, the fungal root colonization within the roots only varied when the plants were subjected to drought stress and the FL inoculum was applied (Figure 2B).

A total of 9 OTUs in the roots of all analyzed samples were found (Figure 2C). Only one of them, OTU1, likely affiliated to an undescribed species from the genus *Dominikia*, was found in all treatments. In addition, this OTU1 resulted to be dominant in all samples except in roots grown on GZ soil with the GZ inoculum and cultivated under well-watered conditions. Certain OTUs were detected exclusively in one of the root treatments related to a specific water regime or soil-inoculum association. Maximum AM fungal diversity values corresponded to well-watered treatments and if AM fungal inoculum was applied to its original soil, while the lowest diversity (0) was found in roots involving FL inoculum on GZ soil under well-watered conditions or in FL soil under drought conditions (Figure 2C).

*Leaf stomatal conductance ( $g_s$ ), relative water content (RWC), photochemical efficiency of photosystem II ( $\Phi PSII$ ), chlorophyll content, and root hydraulic conductivity ( $Lp_r$ )*

Drought treatment caused a massive reduction of  $g_s$  in all treatments except in non-inoculated plants growing in FL soil, as their initial values were already very low (Figure 3A). In FL soil, AM inoculation significantly increased  $g_s$  under well-watered conditions, especially with the GZ inoculum (Fig. 3A). This massive reduction of  $g_s$  by drought could indicate a strong drought stress.

Leaf RWC and  $\Phi PSII$  were reduced by the drought treatment ( $p < 0.0001$ , Table 3), with no effect of the different inoculation treatments or soil (Table 3). Leaf chlorophyll contents were significantly reduced by drought in GZ soils in non-inoculated plants or those inoculated with GZ-AM fungi, as well as in non-inoculated plants growing in FL soil

(Table 3). Under drought condition, AM inoculation (with both GZ and FL) significantly increased chlorophyll contents of plants growing in FL soil (Table 3).

The trends observed in  $g_s$  were not the same as the trends found in root hydraulic conductivity ( $L_{pr}$ ) (Figure 3), in fact a significant negative correlation was found between  $g_s$  and  $L_{pr}$  ( $R = -0.320$ ;  $p = 0.0249$ ). Our study showed that the plants having higher  $L_{pr}$  values were found in FL soil, with a significant increase under drought in plants inoculated with GZ-AM fungi (Figure 3B). However, in FL soil both AM inocula reduced  $L_{pr}$  under well watered conditions. In GZ soil, we did not observed any effects on  $L_{pr}$  by any treatment (Figure 3B)

#### *Molecular identification, expression and abundance of olive aquaporins*

From the molecular approach used, nine new olive aquaporins were described: *OePIP1;2*, *OePIP1;3*, *OePIP2;2*, *OePIP2;3*, *OePIP2;4*, *OePIP2;5*, *OePIP2;6*, *OeTIP1;2*, and *OeTIP1;3*. A phylogenetic tree was built with the currently known PIPs and TIPs aquaporins (*OePIP1;1*, *OePIP2;1*, and *OeTIP1;1*) from *O.europaea* and Poplar (Figure 4).

We next proceeded to analyze expression in the roots of the twelve aquaporins (the three already described and the nine new ones). RNA accumulation could not be detected for the previously known aquaporins *OePIP1;1* and *OePIP2;1* using Q-RT-PCR analyses. To analyze aquaporin expression we first ran an examination of principal components to elucidate which aquaporins were more related with the  $L_{pr}$  and contributed the most to water transport (Figure 5A). We found that expression of *OePIP1;2*, *OePIP2;2*, *OePIP2;3*, *OePIP2;5*, *OePIP2;6* and *OeTIP1;1* were related to whole  $L_{pr}$ , while the expression of aquaporins *OePIP1;3*, *OePIP2;4*, *OeTIP1;2*, and *OeTIP1;3* explained the higher sources of variation within the data. At the same time, soil mycelial hyphal length and proportion of root length colonized also are closely related to  $L_{pr}$ . Stomatal conductance ( $g_s$ ) also

explained the variation observed in  $Lp_r$  data. We ran Pearson correlations with the different aquaporins and  $Lp_r$ , and found that  $Lp_r$  was correlated positively with *OePIP1;2* and *OeTIP1;2*; and negatively correlated with *OePIP1;3*, *OePIP2;4*, and *OeTIP1;3* (Table 3).

ANOVA analyses were run to detect significant differences between treatment means at the different treatments considered. The ANOVA p-values showed, as in previous studies (Barzana et al. 2014), that the expression of all the aquaporins did not follow the same trend at the different treatments considered (Table4-ANOVA p-values, and Supplemental FileS3). However, plants growing in FL soil showed a positive increase in *OeTIP1;3* mRNA accumulation under drought conditions (Figure 5B).

#### *Protein analyses*

The PIP1 proteins were undetected with the methodology applied. Amounts of the PIP2 proteins, as well as the different phosphorylated proteins changed as with the different treatments. The ANOVA p-values showed significant differences between treatment means for all the proteins studied (Table 4). We also ran Pearson's correlations to test possible links between our  $Lp_r$  data and the PIP2 protein abundance and phosphorylation state. It was found a general positive correlation between  $Lp_r$  and the abundance of PIP2 proteins, but a negative correlation with the abundance of phosphorylated PIP2<sub>Ser280</sub> and PIP2<sub>Ser280-283</sub> proteins (Table 4). However, at the treatment level, the higher values of  $Lp_r$  in drought plants in FL soil inoculated with the native mycorrhizas from GZ was not correlated with the abundance of PIP2 proteins (Figure 6A) or the phosphorylation of these proteins at PIP2<sub>Ser280</sub> (Figure 6B), but with the increase on the abundance of phosphorylated proteins PIP2<sub>Ser283</sub>, and PIP2<sub>Ser280-283</sub> (Figure 6C, D).

#### **Discussion**

Olive plants were generated from explants of mature olive trees growing in a typically Mediterranean area. The plants were grown for seven months into two contrasting Mediterranean soils and were inoculated with fungal communities obtained from those soils. In addition, plants were subjected to well-watered and drought stress conditions for four weeks. Under well-watered conditions, the plants growing on GZ soils and inoculated with the GZ fungal community were the only ones which increased their relative height during the four weeks of the stress period. It is well-known that AM fungi can exhibit a considerable level of selectivity in their association with different plants species or plant ecological groups (Öpik et al. 2009, Varela-Cevero et al. 2015). However, these studies have not related the AM associations with the physical and chemical characteristics of the soil, only with the presence of certain AM fungi species. In our study, the higher relative heights of the plants were obtained with fungi from GZ and soils also from GZ, which have the highest content in organic matter, K, P and N. It has been previously found that soil chemistry influences soil microbial community composition, diversity and activity (Ehrenfeld et al. 2005). Greater soil fertility (NPK) increases bacterial biomass and activity in grassland mesocosms and these effects interact with plant species identity in some systems (Innes et al. 2004). Thus, effects of soil chemistry are often system-dependent. Whether or how these soil chemistry effects may interact with plant species identity or plant relatedness is not generally known. In our study, it seems to be crucial for the development of the AM fungi within the roots (that was similar in both well-watered and drought stressed plants), as well as, with the development of the external mycelia within the soil. This combination had also the higher diversity in the OTUs found within the roots under well-watered conditions.

It is also remarkable that plants growing in FL soil without inoculation had the lowest leaf FW, root FW and chlorophyll content, but these values were increased by AM

1 inoculation. These results confirm that under some environmental conditions AM fungi are  
2 essential for plant growth and development (Estrada et al. 2013). Moreover, these plants,  
3 even under well watered conditions, had  $g_s$  values similar to plants under drought stress  
4 conditions. The cause could be the lower values of leaf P and K content. It is known that  
5 nutrient deficiency, including P and K, causes reduction of stomatal conductance (Flores et  
6 al. 2015). In fact, we found a positive correlation between leaf P and K contents and  $g_s$  ( $p$   
7  $< 0.05$ ). Also, no significant correlation was found between any leaf nutrient content and  
8  $L_{p_r}$ , except a negative correlation ( $P > 0.05$ ) with K leaf contents, since plants from FL soil  
9 had less K contents but higher  $L_{p_r}$  values.

10 After four weeks of drought treatment, there was a general reduction in growth and  
11  $g_s$  in all treatments. These are typical responses of plants exposed to severe drought stress.  
12 However, there were some responses of plants that reinforce the previous idea of fungal  
13 and soil chemistry action on roots. We found that plants lost less leaves when growing in  
14 the GZ soils in combination with GZ-inocula, in agreement with the previous idea of the  
15 advantages of certain communities in soils of a certain chemistry and structure (Burns et al.  
16 2015). Olive trees are known to have a tight control of  $g_s$  under different watering regimes  
17 (Torres-Ruiz et al. 2013), as we found under drought stress conditions regardless of the soil  
18 or fungal community applied. This control of  $g_s$ , did not hamper the different responses in  
19  $L_{p_r}$ , as these plants seem to have a suitable hydraulic efficiency to yield water potentials  
20 that maintains the photosynthetic apparatus hydrated under different water demands  
21 (Raimondo et al. 2009). An increase of  $L_{p_r}$  under drought conditions was only found in the  
22 FL soil, and in plants inoculated with the GZ inocula, being this time the dominant fungi  
23 present in the roots the OTU1 (*Dominikia* sp.) in conjunction with OTU5 (*Funneliformis*  
24 sp.). Also, plants growing on FL soil had a higher *OeTIP1;3* expression and higher  
25 phosphorylated  $PIP2_{Ser280}$  and  $PIP2_{Ser280/283}$ . Plant and fungal aquaporin expression,

1 abundance and phosphorylation state can play a major role in root water transport (El-  
2 Mesbahi et al. 2012). Plant aquaporins are usually responsible for the majority of radial  
3 root water transport under severe drought conditions (Bárzana et al. 2012). There are many  
4 studies trying to understand how the presence of AM fungi regulates plant aquaporins  
5 (Bárzana et al. 2014). It is known that AM symbiosis results in altered rates of water  
6 transfer in-and-out of the host plants (Augé 2001), and that also modifies  $L_{pr}$  (Bárzana et  
7 al. 2014, Calvo-Polanco et al. 2014a, Sánchez-Romera et al. 2016). Aquaporins provide a  
8 low resistance pathway for the movement of water across membranes. Furthermore, as  
9 aquaporins can be gated, this provides greater control for the movement of water along  
10 plant tissues (Nyblom et al. 2009, Maurel et al. 2015). PIP and TIP isoforms have been  
11 recognized as central pathways for transcellular and intracellular water transport (Maurel et  
12 al. 2015). Thus, it seems likely that mycorrhizal symbiosis causes significant changes in  
13 aquaporin activity of host plants by changes in their phosphorylation status Calvo-Polanco  
14 et al. 2014a, Sanchez-Romera et al. 2016). On the other hand, it has also been suggested  
15 that water could be absorbed by the external AM mycelium and delivered to the cortical  
16 apoplast, at the symbiotic interfaces, where it would join water taken up via the root  
17 apoplastic pathway (Smith et al., 2010; Barzana et al. 2012). Hyphal water uptake and  
18 transfer to the host plants has been demonstrated in several studies (Marulanda et al., 2003;  
19 Khalvati et al., 2005; Ruth et al. 2011). The increased water uptake by hyphae will be  
20 critical when soil dries and water is retained in the smaller pores where fungal hyphae can  
21 grow (Marulanda et al. 2003), although the role of this hyphae in relation to the internal  
22 fungal development is not very well-known yet under severe drought stress. However, a  
23 close relation between  $L_{pr}$  and mycorrhizal hyphal growth in both soil and roots was  
24 observed. Aroca et al. (2009) observed that the expression of one AM fungal aquaporin  
25 gene was higher in anti-sense tobacco plants with lower expression of tobacco aquaporins

1 than in wild-type plants. This compensatory mechanism could also explain the negative  
2 correlation found here between  $L_{p_r}$  and the expression and phosphorylation state of some  
3 olive aquaporins, where the AM fungal aquaporins could be the main pathway for water  
4 uptake.  
5 Most recently, Tataranni et al. (2015) found that  $L_{p_r}$  in olive trees subjected to drought  
6 stress was close correlated with the amount of suberin and root cell density. It is know that  
7 AM fungi may increase lignin contents of colonized roots, especially in endodermis cells  
8 (Dehne and Schonbeck 1979). Also, most recently, Almeida-Rodríguez et al. (2016) found  
9 that AM symbiosis modifies xylem vessels diameter in *Salix purpurea* plants, modifying  
10 also  $L_{p_r}$  values under copper stress conditions. Anatomical changes caused by AM  
11 symbiosis in olives plants cannot be ruled out, and they deserve further investigations. At  
12 the same time,  $g_s$  explain much of the variation observed in  $L_{p_r}$ , and a significant ( $p<0.05$ )  
13 negative correlation between  $g_s$  and  $L_{p_r}$  was found. Such negative correlation could be  
14 indicative of a preferential water transport through the cell-to-cell path in olive trees, since  
15 this path is not dependent on leaf transpiration (Steudle & Peterson, 1998).

16 The contribution of the different aquaporins studied was addressed with their  
17 *mRNA* expression and protein abundance. The combined expression of different *PIP1*,  
18 *PIP2* and *TIP1* aquaporins (Figure 5A) gave as a combined  $L_{p_r}$  response of the plant to the  
19 different treatments, and each of them responding differently to the several factors that  
20 were affecting the plants (Table 4). Different aquaporins are either up-regulated or down-  
21 regulated by the same stress and those in the same subgroup may have distinct expression  
22 patterns under different stress conditions (Guo et al. 2006; Barzana et al. 2014). The  
23 contribution of all these aquaporins to  $L_{p_r}$  allowed a fine regulation of water uptake under  
24 the GZ soils (Figure 3B) and contributed to the increase of  $L_{p_r}$  under drought conditions in  
25 FL soil with GZ inocula. Among all studied aquaporins, the one that clearly increased

under drought stress was *OeTIP1;3*. The interest on the role of TIPS in water transport has increased in the last few years. Wang et al. (2014) showed that the expression of *TsTIP1;2* increased in response to various stresses in *T. salsuginea*, and ectopic over expression of *TsTIP1;2* enhanced tolerance to drought, salt and oxidative stresses in transgenic Arabidopsis. Most interesting are the results of Henry et al. (2012) who found a good correlation between *TIP* expression and  $Lp_r$  in rice roots subjected to drought stress and by Kuwagata et al. (2012) in rice roots under different air humidity conditions. Also, Boursiac et al. (2005) found that the decrease of  $Lp_r$  caused by salt stress in Arabidopsis was correlated with a down regulation of PIP and TIP expression as well to a relocalization to internal membranes. On the other hand, the effect of the phosphorylation of PIP2 proteins at Ser<sub>283</sub> residue may have contributed to the increase of water uptake in olive plants. Aquaporin phosphorylation is considered as one of the major processes affecting water transport (Suga and Maeshima 2004) and may have also contribute to the adaptation of plant to different stresses, including drought.

Our results show negative correlations between the expression and abundance of some aquaporins and  $Lp_r$ . Similar results were found by Sutka et al. (2011) analyzing the natural variation in  $Lp_r$  of different accessions of Arabidopsis. Most recently, Li et al. (2016) also found a negative correlation between  $Lp_r$  and the gene expression of some Arabidopsis PIPs (*AtPIP1;4* and *AtPIP2;6*). It is known that aquaporins can interact each other with positive or negative results in terms of membrane water permeability, mostly because internalization processes (Bellati et al. 2010; Chaumont and Tyerman 2014). Recently, Hachez et al. (2014) found that the plasma membrane amount of *ZmPIP2;7* proteins is regulated by autophagic degradation when interact with tryptophan-rich sensory protein/translocator. Also, it has been shown that aquaporin *mRNA* expression does not always matches aquaporin protein abundance (Marulanda et al. 2010).



1           In conclusion, we have confirmed that the different characteristics of the soil affect  
2 the development of plants and their responses to drought stress. Also, we have established  
3 that the fitness among AM fungal communities, plant species and soil origin is crucial for  
4 the development of a proper plant-fungus symbiosis. In our case, the inocula from GZ soils  
5 had a higher impact in olive trees under well-watered conditions and also these inocula had  
6 a higher impact in FL soils under drought treatment. So, the AM fungi originated from a  
7 wet climate had a better performance with olive plants under all conditions than the fungi  
8 coming from a more arid environment. The presence of these fungi may have been critical  
9 to modulate plant aquaporin expression, phosphorylation and abundance and hence, for  
10 root water transport. The role of *OeTIP1;3* under water stress may not be related with the  
11 bulk transport of water in plants but may play a crucial role in root osmoregulation of  
12 plants, being part of the fine regulated system of plants water balance.

#### 14 **Acknowledgements**

15 We would like to thank Sonia Molina, Jose Luis Manella and Olga M. López for their  
16 technical support. The study was supported by the Ministry of Economy and  
17 Competitiveness of Spain (Juan de la Cierva Program) and Junta de Andalucía (P10-CVI-  
18 5920 project) for research funding.

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Table 1. Elemental analyses of Grazalema and Freila soils.

Location	pH (Ext 1:2.5)	EC (1:5) (mS/ cm)	P (mg/k g)	K (mg/k g)	Ca (mg/k g)	Mg (mg/ kg)	CaCO <sub>3</sub> (%)	AC (% CaCO <sub>3</sub> )	C (%)	OM (%)	N (%)	C/N	N-NO <sub>3</sub> (mg/l)	N-NH <sub>4</sub> (mg/l)	Amonium (Ext. KCl 2N; mg/kg)	Cl (mg/l)
Grazalema	8.33	0.08	4.65	221.50	4785	450	53.30	8.55	0.83	1.44	0.12	6.99	1.41	0.47	4.65	2.30
Freila	8.98	0.06	<1	45.50	3490	317	34.40	8.10	0.41	0.71	0.05	8.39	0.57	0.35	3.50	2.25

EC: Electrical conductivity; AC: Active Carbonates; OM: Organic Material

Table 2. Nutrient content of leaves of *O.europaea* plants growing into two different types of soils (Grazalema-GZ and Freila-FL), inoculated with different mycorrhizal communities from GZ and FL soils and cultivated under well-watered conditions or subjected to drought stress for four weeks. ANOVA analyses plus post-hoc Tukey's test were run when significant three-way interaction p-values allowed it (only in the case of K contents), and then different letters mean significant differences ( $p < 0.05$ ) among treatments in the same row. When the three-way interaction was non-significant, t-test was used to indicate significant differences ( $p < 0.05$ ) between treatment means at the same column indicated by an asterisk.

		Grazalema Soil			Freila Soil		
		No Myc	Myco GZ	Myco FL	No Myc	Myco GZ	Myco FL
N (mg g <sup>-1</sup> )	Well-Watered	1,09 ± 0,12	1,21 ± 0,03*	1,10 ± 0,12	0,83 ± 0,01	0,80 ± 0,03	0,94 ± 0,03
	Drought	0,92 ± 0,07	1,02 ± 0,02	1,03 ± 0,03	0,85 ± 0,02	0,85 ± 0,04	0,88 ± 0,03
P (mg g <sup>-1</sup> )	Well-Watered	1,09 ± 0,17*	2,12 ± 0,11*	1,12 ± 0,18	0,53 ± 0,01	0,87 ± 0,08	1,16 ± 0,05
	Drought	0,65 ± 0,12	1,23 ± 0,04	1,03 ± 0,11	0,57 ± 0,06	0,73 ± 0,08	1,01 ± 0,16
K (mg g <sup>-1</sup> )	Well-Watered	7,37 ± 0,68 <b>ab</b>	7,99 ± 0,36 <b>a</b>	8,03 ± 0,46 <b>a</b>	6,02 ± 0,25 <b>cde</b>	6,89 ± 0,43 <b>abc</b>	7,86 ± 0,11 <b>a</b>
	Drought	6,03 ± 0,32 <b>cde</b>	6,28 ± 0,34 <b>bcd</b>	5,71 ± 0,51 <b>de</b>	5,02 ± 0,19 <b>e</b>	4,99 ± 0,25 <b>e</b>	5,63 ± 0,57 <b>de</b>
Mg (mg g <sup>-1</sup> )	Well-Watered	1,22 ± 0,18	1,33 ± 0,19	1,14 ± 0,12	1,30 ± 0,08	1,28 ± 0,12	1,15 ± 0,05
	Drought	1,07 ± 0,12	1,01 ± 0,05	1,16 ± 0,03	1,36 ± 0,26	1,26 ± 0,15	1,20 ± 0,16
S (mg g <sup>-1</sup> )	Well-Watered	0,96 ± 0,07	1,08 ± 0,05	1,12 ± 0,18	0,87 ± 0,05	0,92 ± 0,06	0,92 ± 0,06
	Drought	0,81 ± 0,04	0,92 ± 0,04	1,03 ± 0,11	0,70 ± 0,02	0,92 ± 0,03	0,88 ± 0,03
Ca (mg g <sup>-1</sup> )	Well-Watered	10,11 ± 0,95	11,32 ± 1,48	10,95 ± 1,49	8,70 ± 1,76	10,50 ± 0,96	8,54 ± 0,25
	Drought	9,15 ± 1,1	9,07 ± 0,42	10,37 ± 0,18	7,75 ± 0,43	11,20 ± 0,51	10,22 ± 1,22

	Soil	Myc	Soil x Myc	Drought	Soil x Drought	Myc x Drought	Soil x Myc x Drought
N	<.0001	0.8919	0.0290	0.2833	0.0019	0.3662	0.0744
P	<.0001	<.0001	0.0098	0.0002	0.0034	0.2691	0.4605
K	0.0010	0.0613	0.1147	<.0001	0.1150	0.0019	0.0455
Mg	0.1992	0.7224	0.7163	0.4618	0.2672	0.5663	0.7945

S	0.0027	0.0216	0.7647	0.1420	0.1032	0.4277	0.3421
Ca	0.2627	0.0951	0.2972	0.8970	0.0535	0.6630	0.9349

ANOVA p-values for the different parameters measured: Soil- soil type; Myc – Non-inoculated, inoculated with AM from GZ or inoculated with AM from FL; and Well-watered and drought stress.

Table 3. Leaf and root fresh weight (FW), leaf relative water content (RWC), photochemical efficiency of photosystem II ( $\Phi$ PSII) and chlorophyll content in *O.europaea* plants growing into two different types of soils (Grazalema-GZ and Freila-FL), inoculated with different mycorrhizal communities from GZ and FL soils and cultivated under well-watered conditions or subjected to drought stress for four weeks. As the three-way interaction was non-significant for any parameter, t-test was used to indicate significant differences ( $p < 0.05$ ) between treatment means at the same column indicated by an asterisk ( $n=6$ ).

		Grazalema Soil			Freila Soil		
		No Myc	MycoGZ	Myco FL	No Myc	MycoGZ	Myco FL
Leaf FW (g)	Well-watered	5.6 $\pm$ 1.0*	7.1 $\pm$ 0.8	5.9 $\pm$ 0.7*	2.7 $\pm$ 0.4	3.6 $\pm$ 0.3	3.9 $\pm$ 0.6
	Drought	3.4 $\pm$ 0.4	5.9 $\pm$ 0.8	3.1 $\pm$ 0.8	2.6 $\pm$ 0.5	3.0 $\pm$ 0.6	2.9 $\pm$ 0.5
Root FW (g)	Well-watered	4.7 $\pm$ 0.9	4.5 $\pm$ 0.3	5.0 $\pm$ 1.1	3.0 $\pm$ 0.7*	5.1 $\pm$ 0.8*	4.8 $\pm$ 0.7*
	Drought	3.3 $\pm$ 0.8	4.0 $\pm$ 0.5	3.8 $\pm$ 1.34	1.5 $\pm$ 0.4	3.3 $\pm$ 0.5	2.5 $\pm$ 0.8
$\Phi$ PSII (r.u)	Well-watered	0.61 $\pm$ 0.01*	0.61 $\pm$ 0.01*	0.60 $\pm$ 0.01*	0.54 $\pm$ 0.10*	0.57 $\pm$ 0.05*	0.58 $\pm$ 0.06*
	Drought	0.16 $\pm$ 0.07	0.18 $\pm$ 0.04	0.19 $\pm$ 0.07	0.25 $\pm$ 0.02	0.30 $\pm$ 0.07	0.31 $\pm$ 0.05
RWC(%)	Well-watered	94 $\pm$ 1*	92 $\pm$ 1*	94 $\pm$ 1*	94 $\pm$ 1*	94 $\pm$ 1*	91 $\pm$ 1*
	Drought	85 $\pm$ 1	85 $\pm$ 2	90 $\pm$ 1	90 $\pm$ 1	85 $\pm$ 3	86 $\pm$ 3
Chlorophyll content (mg g <sup>-1</sup> )	Well-watered	7.4 $\pm$ 1.1*	7.3 $\pm$ 0.2*	6.4 $\pm$ 0.5	5.3 $\pm$ 0.7*	6.1 $\pm$ 0.7	6.5 $\pm$ 0.3
	Drought	4.7 $\pm$ 0.2	5.3 $\pm$ 0.4	4.8 $\pm$ 1.6	3.8 $\pm$ 0.2	5.0 $\pm$ 0.6	6.3 $\pm$ 0.3

	Soil	Myc	Soil x Myc	Drought	Soil x Drought	Myc x Drought	Soil x Myc x Drought
Leaf FW	<.0001	0.0100	0.0630	0.0008	0.0534	0.5122	0.6709
Root FW	0.0441	0.0604	0.2186	0.0010	0.3005	0.8372	0.7938
$\Phi$ PSII	0.4322	0.5239	0.9134	<.0001	0.0296	0.7099	0.8641

RWC	0.7804	0.3389	0.1711	<.0001	0.9200	0.3983	0.3298
Chlorophyll	0.0346	0.0377	0.0015	<.0001	0.0154	0.1724	0.8686

ANOVA p-values for the different parameters measured: Soil- soil type; Myc – Non-inoculated, inoculated with AM from GZ or inoculated with AM from FL; and Drought- Well-watered and drought stress.

Table 4. Pearson correlations between root hydraulic conductivity ( $L_{pr}$ ) and the different aquaporin expression determined as well as the PIP2 root protein abundance and phosphorylation state (PIP2<sub>280</sub> - proteins phosphorylated at Ser-280; PIP2<sub>283</sub>- proteins phosphorylated at Ser-283; and PIP2<sub>280-283</sub> proteins phosphorylated at both Ser-280 and Ser-283).

	<i>OePIP1;2</i>	<i>OePIP1;3</i>	<i>OePIP2;2</i>	<i>OePIP23</i>	<i>OePIP2;4</i>	<i>OePIP2;5</i>	<i>OePIP2;6</i>	<i>OeTIP1;1</i>	<i>OeTIP1;2</i>	<i>OeTIP1;3</i>	PIP2	PIP2 <sub>280</sub>	PIP2 <sub>283</sub>	PIP2 <sub>280-283</sub>
$L_{pr}$	0.357	-0.378	0.208	-0.099	-0.328	-0.219	0.013	-0.153	0.492	-0.401	0.399	-0.524	0.084	-0.509
	0.0219*	0.0125*	0.1860	0.5342	0.0417*	0.1869	0.9418	0.3603	0.0013*	0.0070*	0.0089*	0.0002*	0.5831	0.0004*



Table 5. ANOVA p-values for expression of 10 *Olea europaea* aquaporins and the PIP2 root protein abundance and phosphorylation state (PIP2<sub>280</sub>- proteins phosphorylated at Ser-280; PIP2<sub>283</sub>- proteins phosphorylated at Ser-283; and PIP2<sub>280-283</sub> proteins phosphorylated at both Ser-280 and Ser-283).

	Soil	Myc	Soil x Myc	Drought	Soil x Drought	Myc x Drought	Soil x Myc x Drought
<i>OePIP1;2</i>	0.6948	0.6960	0.2279	0.0099*	0.7861	0.2295	0.5441
<i>OePIP1;3</i>	0.0247*	0.0028*	0.0002*	0.1258	0.1597	0.0746	0.0482*
<i>OePIP2;2</i>	0.9796	0.0077*	0.0026*	<.0001*	0.4775	0.0075*	0.1077
<i>OePIP2;3</i>	0.0369*	0.1614	0.4516	0.5105	0.9305	0.1303	0.0443*
<i>OePIP2;4</i>	0.5710	0.0143*	0.4131	0.0005*	0.9357	0.1137	0.0606
<i>OePIP2;5</i>	0.0243*	0.1782	0.0133*	0.7510	0.0898	0.1810	0.1267
<i>OePIP2;6</i>	0.6627	0.6126	0.3996	0.3079	0.0066*	0.0271*	0.0167*
<i>OeTIP1;1</i>	0.5439	0.6830	0.4257	0.6290	0.8697	0.5195	0.6225
<i>OeTIP1;2</i>	0.9458	0.6177	0.0060*	0.0037*	0.8828	0.8179	0.0203*
<i>OeTIP1;3</i>	0.9647	0.1395	0.0536	<.0001*	0.0015*	0.3041	0.5517
PIP2	0.0358	0.5679	0.0453	0.9705	0.0002*	0.7366	0.0061*
PIP2 <sub>280</sub>	0.0007*	<.0001*	0.0104*	<.0001*	0.0271*	<.0001*	<.0001*
PIP2 <sub>283</sub>	0.6411	0.1466	0.0887	0.5913	0.0380*	0.5233	0.0162*
PIP2 <sub>280/283</sub>	0.6581	0.0067*	0.0112*	<.0001*	0.0061*	<.0001*	<.0001*

## Figure Captions

Figure 1. Relative plant height (A), relative number of leaves (B), and ANOVA p-values table, in *O.europaea* cv. Picual plants growing into two different types of soils (Grazalema-GZ and Freila-FL), and inoculated with different mycorrhizal communities from GZ and FL soils. The plants were either cultivated under well-watered conditions or subjected to drought stress for four weeks. Asterisks above bars indicate significant differences ( $p<0.05$ ) between well-watered and drought treatments after t-student test ( $n=6$ ).

Figure 2. Soil hyphal length (A), percentage of mycorrhizal root length (B) and root mycorrhizal diversity (C) in *O. europaea* cv. Picual plants growing into two different types of soils (Grazalema-GZ and Freila-FL), and inoculated with different mycorrhizal communities from GZ and FL soils. The plants were either cultivated under well-watered conditions or subjected to drought stress for four weeks. Different letters means significant differences ( $p<0.05$ ) among treatments after ANOVA and Tuckey's test ( $n=4$ ). The numbers above the columns in panel C are the Shannon biodiversity index and the numbers under brackets are the Specific Richness.

Figure 3. Stomatal conductance (A) and root hydraulic conductivity (B) in *O.europaea* cv. Picual plants growing into two different types of soils (Grazalema-GZ and Freila-FL), and inoculated with different mycorrhizal communities from GZ and FL soils. The plants were either cultivated under well-watered conditions or subjected to drought stress for four weeks. Different letters means significant differences ( $p<0.05$ ) among treatments after ANOVA and Tukey's test ( $n=6$ ).

Figure 4. Phylogenetic tree of the PIP and TIP aquaporins from *Populus trichocarpa* (Gupta and Sankaramakrishnan 2009) and *Olea europaea* (Secchi et al. 2007; present study) species. The aquaporins of the two plant species were analyzed with MEGA4 software using neighbor-joining method. The distance scale represents evolutionary distance, expressed in the number of substitutions per amino acid.

Figure 5. Principal component analyses (A) for the expression of 10 aquaporin genes, PIP1 and PIP2 abundances, PIP2 phosphorylation state, root hydraulic conductivity ( $L_{pr}$ ), stomatal conductance ( $gs$ ), root colonization rate (MycP) and soil hyphal length (MycL). Relative *mRNA* expression of *OeTIP1;3* (B) in roots of *O.europaea* cv. Picual plants growing into two different types of soils (Grazalema-GZ and Freila-FL), and inoculated with different mycorrhizal communities from GZ and FL soils. The plants were either cultivated under well-watered conditions or subjected to drought stress for four weeks. Asterisks above bars indicate significant differences ( $p<0.05$ ) between well-watered and drought treatments after t-student test ( $n=3$ ).

Figure 6. Quantification of PIP2 proteins (A), PIP2 phosphorylated proteins at Ser-280 (B), Ser-283 (C) and both Ser-280 and 283 (D) in roots of *O. europaea* cv. Picual plants growing into two different types of soils (Grazalema-GZ and Freila-FL), and inoculated with different mycorrhizal communities from GZ and FL soils. The plants were either

cultivated under well-watered conditions or subjected to drought stress for four weeks  
Different letters means significant differences ( $p < 0.05$ ) among treatments after ANOVA  
and Tukey's test ( $n=3$ ).